Protein determination on the basis of copper-binding capacity

Current photometric methods for the determination of protein rely either on the special properties of aromatic residues[1–3] or solely on the presence of polypeptide structures[4–6]. Those procedures dependent on aromatic groups are sensitive but vary in the absolute values given by different proteins. The procedures dependent on the complexing of copper by polypeptides, the so-called biuret methods[4,5], are more reliable but much less sensitive. NEILSEN's micro procedure[6] based on the biuret reaction has not been found generally useful in this laboratory because of turbidity, caused by precipitation of the water-insoluble copper diethyldithiocarbamate[7], and highly colored blanks, apparently caused by the formation of soluble copper-phosphate complexes[8]. This communication reports a rapid new procedure utilizing the biuret reaction, which has the reliability of the biuret method and a useful range of 0.05–1.0 mg protein.

Reagents

(a) Alkaline copper tartrate reagent: 10 ml 0.20 *M* $CuSO_4$ are added to 590 ml 0.167 *N* NaOH containing 2.0 g potassium sodium tartrate tetrahydrate. (b) Dowex-1 suspension: Bio-Rad AG1-X8, 200–400 mesh, chloride form*, is suspended in deionized water at a density of 200 mg/ml. (c) Color reagent: Sodium diethyldithiocarbamate is dissolved at a concentration of 1.0 mg/ml in a 0.025 % solution of crystalline bovine serum albumin. All of the reagents are stable for many weeks at room temperature.

Procedure

The protein solution to be analyzed is freed of salts and free amino acids by dialysis or the protein is precipitated with 0.5 vol. of 3 *M* trichloracetic acid. The concentration of salts and amino acids in the sample to be precipitated must be less than 0.4 *M*. Above this level, interference is most pronounced at the lower protein concentrations. Agreement between assay values obtained with aliquots of different sizes assures the absence of interference. The precipitated protein is sedimented by centrifugation for 5 min at 20,000 × *g* and then resuspended in 0.50 ml of deionized water. The protein suspension or 0.50 ml of dialyzed protein solution, containing 0.05–1.0 mg protein, is mixed in a centrifuge tube with 1.00 ml of alkaline copper tartrate solution, in which the protein precipitates readily dissolve. After at least 5 min at room temperature, 1.0 ml of Dowex-1 suspension is added, followed by 5.0 ml of color reagent. After mixing, the tube is centrifuged briefly at low speed and the supernatant solution is decanted into a cuvette. Absorbancies are determined at 446 or 486 mμ, depending on the amount of protein present, against a blank containing 2.5 ml of deionized water and 5.0 ml of color reagent. Any resin carried over in decanting settles rapidly and causes no interference with the photometry. The color is stable indefinitely.

Standard curves for the method are given in Figs. 1 and 2. The absorbancy response is linear up to 0.4 mg protein. At 446 mμ, which is the absorption maximum,

* California Corporation for Biochemical Research. No cycling or other preparation of the resin is necessary. Equilibration with alkaline tartrate prior to use diminishes the effects of interfering substances but also reduces the sensitivity of the method.

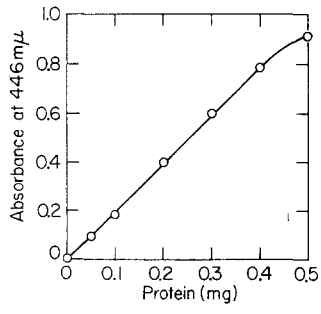

Fig. 1. Standard curve for the procedure at 446 mμ. The points shown are the means of values obtained for the five crystalline proteins of Table I. The instrument used was a Coleman Junior Spectrophotometer.

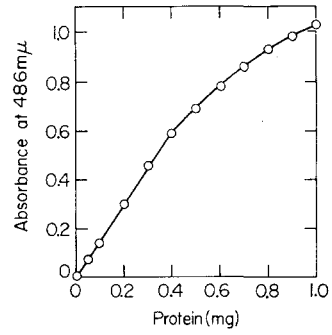

Fig. 2. Standard curve for the procedure at 486 mμ. Other conditions as in Fig. 1.

this corresponds to the most useful range of the photometer. The sensitivity of the method may be increased somewhat by using a smaller volume of a more concentrated color reagent and a suitable photometer.

The reliability of the method with different proteins is indicated by the data given in Table I. As was expected, the new procedure and the biuret method have similar reliabilities, while the sensitivity of the new procedure approaches that of the LOWRY method.

TABLE I

COMPARISON OF FOUR ANALYTICAL PROCEDURES IN THE DETERMINATION OF FIVE CRYSTALLINE PROTEINS

The crystalline proteins used were purchased from Nutritional Biochemicals Corporation. Dry weights were determined by drying samples to constant weight at 30° over Al_2O_3. The absorbancy values given are based on data sufficient to insure random errors smaller than 2 %.

Protein	*Apparent protein content of solutions containing 1.00 mg/ml**			
	*Estimation based on absorbancy at 280 mμ***	LOWRY *method**** *(ref. 3)*	*Biuret method**** *(ref. 5)*	*Present method****
Edestin	0.69	0.91	1.04	1.00
β-lactoglobulin	0.73	0.67	0.93	0.93
Bovine serum albumin	0.52	0.97	0.95	0.97
Pepsin	1.09	1.18	0.94	1.04
Lysozyme	1.97	1.22	1.14	1.05
Mean deviation (as % of mean)	42 %	17 %	7 %	4 %
Sensitivity (absorbance units/mg in the final volumes stated)	0.43	2.6	0.05	2.0

* The mean absorbancy value per mg for each method was taken as the standard for that method.

** Determined using the Beckman Model DU Ultraviolet Spectrophotometer, final vol. 3.0 ml.

*** Determined using the Coleman Junior Spectrophotometer, final vol. 7.5 ml. Absorbancy values were determined at the wavelength of the absorption maximum for each method.

The tartrate in the copper reagent serves to maintain Cu^{++} in solution at alkaline pH, as in the biuret method. Under these conditions the reaction with protein proceeds rapidly. Since the coordination number of Cu^{++} is 4, each Cu^{++} binds two tartrate ions, and the net charge on the copper-tartrate complex is negative. In the same way, each Cu^{++} binds four peptide bond nitrogens, but the net charge on the copper-protein complex is positive. Accordingly, the strong anion exchanger Dowex-1 removes from solution all copper except that bound by protein. In the final step, precipitation of copper diethyldithiocarbamate was found to be prevented by the presence of excess protein, added as albumin in the color reagent. The absence of color in the color reagent itself and the quantitative removal of excess copper by the ion-exchange resin result in colorless blanks. This circumstance assures high sensitivity for small amounts of protein and minimizes errors caused by inaccuracies in procedure.

In summary, a quantitative analytical procedure based on the copper-binding capacity of protein has been developed. Protein-complexed copper is determined colorimetrically with diethyldithiocarbamate after removal of excess copper by an ion-exchange resin. The procedure has 40 times the sensitivity of the biuret method and equal reliability.

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[1] O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
[2] H. KALCKAR, *J. Biol. Chem.*, 167 (1947) 461.
[3] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[4] J. W. MEHL, *J. Biol. Chem.*, 157 (1944) 173.
[5] G. BEIZENHERZ, H. J. BOLTZE, T. BÜCHER, R. CZÖK, K.-H. GARBADE, E. MEYER-ARENDT AND G. PFLEIDERER, *Z. Naturforsch.*, 8b (1953) 555.
[6] H. NEILSEN, *Acta Chem. Scand.*, 12 (1958) 38.
[7] N. CHIEREGO, *Arch. oceanog. e limnol.*, 10 (1955) 197.
[8] J. MERCADIE, *Compt. rend.*, 221 (1945) 58.

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